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(71) Applicant: TRANSKARYOTIC THERAPIES, INC. [US/US]; 195 Albany Street, Cambridge, MA 02139 (US).

(72) Inventors: TRECO, Douglas, A.; 87 Brantwood Road, Arlington, MA 02174 (US). HEARTLEIN, Michael, W.; 167 Reed Farm Road, Boxborough, MA 01719 (US). SELDEN, Richard, F.; 106 Bristol Road, Wellesley, MA 02181 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

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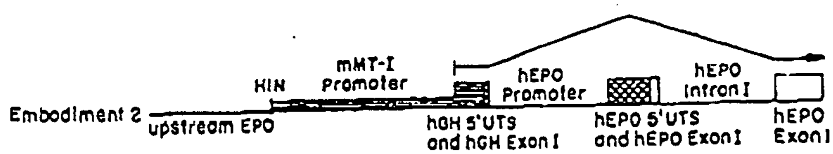

(57) Abstract

Method of activating expression of and amplifying an endogenous gene in genomic DNA of a vertebrate cell which is not expressed in the cell as obtained or is not expressed at significant levels in the cell as obtained, comprising the steps of: (a) transfecting cells with DNA sequences comprising: 1) exogenous DNA sequences which repair, alter, delete or replace a sequence present in the cell or which are regulatory sequences not normally functionally linked to the endogenous gene in the cell as obtained; 2) DNA sequences homologous with genomic DNA sequences at a preselected site in the cells; and 3) amplifiable DNA encoding a selectable marker; (b) maintaining the cells under conditions appropriate for homologous recombination to occur between DNA sequences homologous with genomic DNA sequences and genomic DNA sequences; (c) culturing the homologously recombinant cells produced in (b) under conditions which select from amplification of the amplifiable DNA encoding a selectable marker, whereby the amplifiable DNA encoding a selectable marker and the endogenous gene functionally linked to exogenous DNA of (a) 1) are coamplified.

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ACTIVATING EXPRESSION OF AN AMPLIFYING ENDOGENOUS GENE BY HOMOLOGOUS RECOMBINATION.

Background of the Invention

Current approaches to treating disease by administering therapeutic proteins include *in vitro* production of therapeutic proteins for conventional pharmaceutical delivery (e.g. intravenous, subcutaneous, or intramuscular injection) and, more recently, gene therapy.

Proteins of therapeutic interest are generally produced by introducing exogenous DNA encoding the protein of therapeutic interest into appropriate cells. Presently-available approaches to gene therapy make use of infectious vectors, such as retroviral vectors, which include the genetic material to be expressed. Such approaches have limitations, such as the potential of generating replication-competent virus during vector production; recombination between the therapeutic virus and endogenous retroviral genomes, potentially generating infectious agents with novel cell specificities, host ranges, or increased virulence and cytotoxicity; independent integration into large numbers of cells, increasing the risk of a tumorigenic insertional event; limited cloning capacity in the retrovirus (which restricts therapeutic applicability) and short-lived *in vivo* expression of the product of interest. A better approach to providing gene products, particularly one which avoids the risks associated with presently available methods and provides long-term treatment, would be valuable.

Summary of the Invention

The present invention relates to improved methods for both the *in vitro* production of therapeutic proteins and for the production and delivery of therapeutic proteins by

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gene therapy. The present method describes an approach which activates expression of endogenous cellular genes, and further allows amplification of the activated endogenous cellular genes, which does not require in vitro manipulation and transfection of exogenous DNA encoding proteins of therapeutic interest.

The present invention relates to transfected cells, both transfected primary or secondary cells (i.e., non-immortalized cells) and transfected immortalized cells, useful for producing proteins, particularly therapeutic proteins, methods of making such cells, methods of using the cells for in vitro protein production and methods of gene therapy. Cells of the present invention are of vertebrate origin, particularly of mammalian origin and even more particularly of human origin. Cells produced by the method of the present invention contain exogenous DNA which encodes a therapeutic product, exogenous DNA which is itself a therapeutic product and/or exogenous DNA which causes the transfected cells to express a gene at a higher level or with a pattern of regulation or induction that is different than occurs in the corresponding nontransfected cell.

The present invention also relates to methods by which primary, secondary, and immortalized cells are transfected to include exogenous genetic material, methods of producing clonal cell strains or heterogenous cell strains, and methods of immunizing animals, or producing antibodies in immunized animals, using the transfected primary, secondary, or immortalized cells.

The present invention relates particularly to a method of gene targeting or homologous recombination in cells of vertebrate, particularly mammalian, origin. That is, it relates to a method of introducing DNA into primary, secondary, or immortalized cells of vertebrate origin

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through homologous recombination, such that the DNA is introduced into genomic DNA of the primary, secondary, or immortalized cells at a preselected site. The targeting sequences used are determined by (selected with reference to) the site into which the exogenous DNA is to be inserted. The present invention further relates to homologously recombinant primary, secondary, or immortalized cells, referred to as homologously recombinant (HR) primary, secondary or immortalized cells, produced by the present method and to uses of the HR primary, secondary, or immortalized cells.

The present invention also relates to a method of activating (i.e., turning on) a gene present in primary, secondary, or immortalized cells of vertebrate origin, which is normally not expressed in the cells or is not expressed at physiologically significant levels in the cells as obtained. According to the present method, homologous recombination is used to replace or disable the regulatory region normally associated with the gene in cells as obtained with a regulatory sequence which causes the gene to be expressed at levels higher than evident in the corresponding nontransfected cell, or to display a pattern of regulation or induction that is different than evident in the corresponding nontransfected cell. The present invention, therefore, relates to a method of making proteins by turning on or activating an endogenous gene which encodes the desired product in transfected primary, secondary, or immortalized cells.

In one embodiment, the activated gene can be further amplified by the inclusion of a selectable marker gene which has the property that cells containing amplified copies of the selectable marker gene can be selected for by culturing the cells in the presence of the appropriate selectable agent. The activated endogenous gene which is

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near or linked to the amplified selectable marker gene will also be amplified in cells containing the amplified selectable marker gene. Cells containing many copies of the activated endogenous gene are useful for in vitro protein production and gene therapy.

Gene targeting and amplification as disclosed in the present invention are particularly useful for turning on the expression of genes which form transcription units which are sufficiently large that they are difficult to isolate and express, or for turning on genes for which the entire protein coding region is unavailable or has not been cloned. The present invention also describes a method by which homologous recombination is used to convert a gene into a cDNA copy, devoid of introns, for transfer into yeast or bacteria for in vitro protein production.

Transfected cells of the present invention are useful in a number of applications in humans and animals. In one embodiment, the cells can be implanted into a human or an animal for protein delivery in the human or animal. For example, human growth hormone (hGH), human EPO (hEPO), human insulinotropin and other proteins can be delivered systemically or locally in humans for therapeutic benefits. Barrier devices, which contain transfected cells which express a therapeutic product and through which the therapeutic product is freely permeable, can be used to retain cells in a fixed position in vivo or to protect and isolate the cells from the host's immune system. Barrier devices are particularly useful and allow transfected immortalized cells, transfected cells from another species (transfected xenogeneic cells), or cells from a nonhistocompatibility-matched donor (transfected allogeneic cells) to be implanted for treatment of human or animal conditions or for agricultural uses (e.g., meat and dairy

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production). Barrier devices also allow convenient short-term (i.e., transient) therapy by providing ready access to the cells for removal when the treatment regimen is to be halted for any reason. Transfected xenogeneic and allogeneic cells may be used for short-term gene therapy, such that the gene product produced by the cells will be delivered in vivo until the cells are rejected by the host's immune system.

Transfected cells of the present invention are also useful for eliciting antibody production or for immunizing humans and animals against pathogenic agents. Implanted transfected cells can be used to deliver immunizing antigens that result in stimulation of the host's cellular and humoral immune responses. These immune responses can be designed for protection of the host from future infectious agents (i.e., for vaccination), to stimulate and augment the disease-fighting capabilities directed against an ongoing infection, or to produce antibodies directed against the antigen produced in vivo by the transfected cells that can be useful for therapeutic or diagnostic purposes. Removable barrier devices can be used to allow a simple means of terminating exposure to the antigen. Alternatively, the use of cells that will ultimately be rejected (xenogeneic or allogeneic transfected cells) can be used to limit exposure to the antigen, since antigen production will cease when the cells have been rejected.

The methods of the present invention can be used to produce primary, secondary, or immortalized cells producing a wide variety of therapeutically useful products, including (but not limited to): hormones, cytokines, antigens, antibodies, enzymes, clotting factors, transport proteins, receptors, regulatory proteins, structural proteins, transcription factors, or anti-sense RNA. Additionally, the methods of the present invention can be

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used to produce cells which produce non-naturally occurring ribozymes, proteins, or nucleic acids which are useful for in vitro production of a therapeutic product or for gene therapy.

Brief Description of the Drawings

Figure 1 is a schematic representation of plasmid pXGH5, which includes the human growth hormone (hGH) gene under the control of the mouse metallothionein promoter.

Figure 2 is a schematic representation of plasmid pcDNEO, which includes the neo coding region (BamHI-BglII fragment) from plasmid pSV2neo inserted into the BamHI site of plasmid pcD; the Amp-R and pBR322Ori sequences from pBR322; and the polyA, 16S splice junctions and early promoter regions from SV40.

Figure 3 is a schematic representation of plasmid pXEPO1. The solid black arc represents the pUC12 backbone and the arrow denotes the direction of transcription of the ampicillin resistance gene. The stippled arc represents the mouse metallothionein promoter (pmMT1). The unfilled arc interrupted by black boxes represents the human erythropoietin EPO gene (the black boxes denote exons and the arrow indicates the direction hEPO transcription). The relative positions of restriction endonuclease recognition sites are indicated.

Figure 4 is a schematic representation of plasmid pE3neoEPO. The positions of the human erythropoietin gene and the neo and amp resistance genes are indicated. Arrows indicate the directions of transcription of the various genes. pmMT1 denotes the mouse metallothionein promoter (driving hEPO expression) and pTK denotes the Herpes Simplex Virus thymidine kinase promoter (driving neo expression). The dotted regions of the map mark the positions of human HPRT sequences. The relative positions